DSS-MEDIATED INHIBITION OF QUANTITAVE REAL-TIME POLYMERASE CHAIN REACTION

A Senior Scholars Thesis

by

JASPREET KAUR

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2009

Major: Genetics

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Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

DSS-mediated Inhibition of Quantitative Real-time Polymerase Chain Reaction.
(April 2009)

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The incidence of inflammatory bowel diseases (IBD), including ulcerative colitis (UC), is on the rise. UC is modeled in rodents by the consumption of dextran sodium sulfate (DSS). Quantitative real-time polymerase chain reaction (qPCR) is a sensitive technique used to quantify the abundance of "target gene sequences", including those that may be related to the causation of UC. Using *Lactobacillus*-group-specific primers, qPCR was used to determine the abundance of *Lactobacillus* species found in the feces of both DSS-treated (n=4) and untreated control rats (n=6). DNA isolated from the feces of untreated control rats yielded strong and reproducible qPCR signals, whereas DNA isolated from the feces of DSS-treated rats often failed to produce detectable qPCR signals. We hypothesized that DSS co-purified with the extracted DNA and reduced PCR efficiency by either (i) competing with DNA for the Taq DNA polymerase active site or (ii) binding and modifying the polymerase. To test this hypothesis, we conducted

DSS-spiking studies and employed a number of techniques to remove residual DSS. We confirm here that DSS is indeed responsible for the observed qPCR failure and that the Dneasy Blood and Tissue Kit (Qiagen) was most effective in removing residual DSS and restoring PCR efficiency.

Dedicated to my father,

Paramjit Singh,

without whom my achievements would not have been attained.

ACKNOWLEDGMENTS

I would like to thank Dr. Sturino for providing me with this opportunity. Thank you for your help, your priceless time, guidance and invaluable wisdom. Most of all, thank you for being my mentor. You have given me great advice to succeed in the professional world. I would like to thank Laura Thomas for training me in the laboratory and providing me with the useful skills. Thanks for being understanding and caring. I will always be "your undergrad." Also, I would like to thank Carly Ferguson for all the real-time PCR help.

NOMENCLATURE

C_t	Cycle threshold
dsDNA dye	Double-stranded DNA dye
DSS	Dextran Sodium Sulfate
GI	Gastrointestinal Tract
IBD	Inflammatory Bowel Disease
nm	Nanometers
q-PCR	Quantitative Real-Time Polymerase Chain Reaction
UC	Ulcerative Colitis

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CHAPTER I

INTRODUCTION

1.1 Ulcerative colitis

Ulcerative Colitis (UC) is a chronic inflammatory condition of the large intestine (8). In the period 1984-1993, UC affected only 8 out of 100,000 people in the United States. In 2001, however, the incidence had increased to 246 cases per 100,000 (10). Emerging evidence indicates a positive correlation between the incidences of reoccurring ulcerative colitis and colon cancer (3, 12). The aetiology of the disease is unknown; however, the presence of a luminal commensal microbiota is known to be vital for the initiation and progression of the disease in both chemically-induced and spontaneous models of colitis (7). Dextran Sodium Sulfate (DSS) is a well established model used to mimic human UC in rodents and has been used to study the effects of inflammation on the gastrointestinal (GI) microbiota (11, our unpublished results). Traditionally, culture-based methods have been used to study microbial communities; however, due to limitations of cultivation-dependent strategies, detection of viable but not cultivatable cells have lead to the use of modern culture-independent techniques (9).

1.2 Quantitative real-time polymerase chain reaction

A number of molecular techniques can be used to estimate the number of microbial cells including, randomly amplified polymorphic DNA (RAPD) polymerase chain reaction denaturing gel electrophoresis (PCR-DGGE).

This thesis follows the style of Applied and Environmental Microbiology.

Accurate quantification of DNA can be also be rapidly and reproducibly obtained by quantitative real-time polymerase chain reaction (qPCR) (4). Real-time PCR is a precise and sensitive technique which can detect minute changes by quantifying PCR products in "real-time" and eliminates down-stream processing (14). The qPCR reaction is carried out in a thermocycler equipped with a fluorescence detector. SYBR® Green, which absorbs blue light at (λ_{max}= 488nm) and emits green light at (λ_{max} = 522nm) is a fluorophore and intercalating dye that is commonly used during qPCR (17). When double-stranded DNA (dsDNA) dye, such as SYBR®Green (FIG. 1) bind to the double stranded anti-parallel DNA, the dye emits a fluorescence signal. Therefore, during qPCR reaction, increased fluorescence intensity is positively correlated to an increase in DNA product (14). SYBR® Green binds to DNA non-specifically, meaning that it will bind the intended target amplicons, primer-dimers and unintended or non-specific PCR products. The non-specific binding of the SYBR Green molecule can result in inaccurate quantification of the target (17), however it enables signal quantification in the absence of molecular probes (e.g. Taqman assays).

In qPCR, amplification is detected in the log phase (FIG.2), also known as exponential phase by plotting copy number against cycle number (4). Target Copy number is a measure of the DNA product obtained from amplification. The threshold line, an arbitrary number, is set at a specific copy number at which fluorescence is observed to increase above the background level. The cycle threshold (Ct) value refers to the cycle number at which the fluorescence emitted from the sample crosses the threshold line (3).

C_t values are indirectly related to copy numbers. Hereafter, the target DNA is quantitated against a calibration (standard) curve that connects threshold cycles to exact concentrations of sample DNA (9).

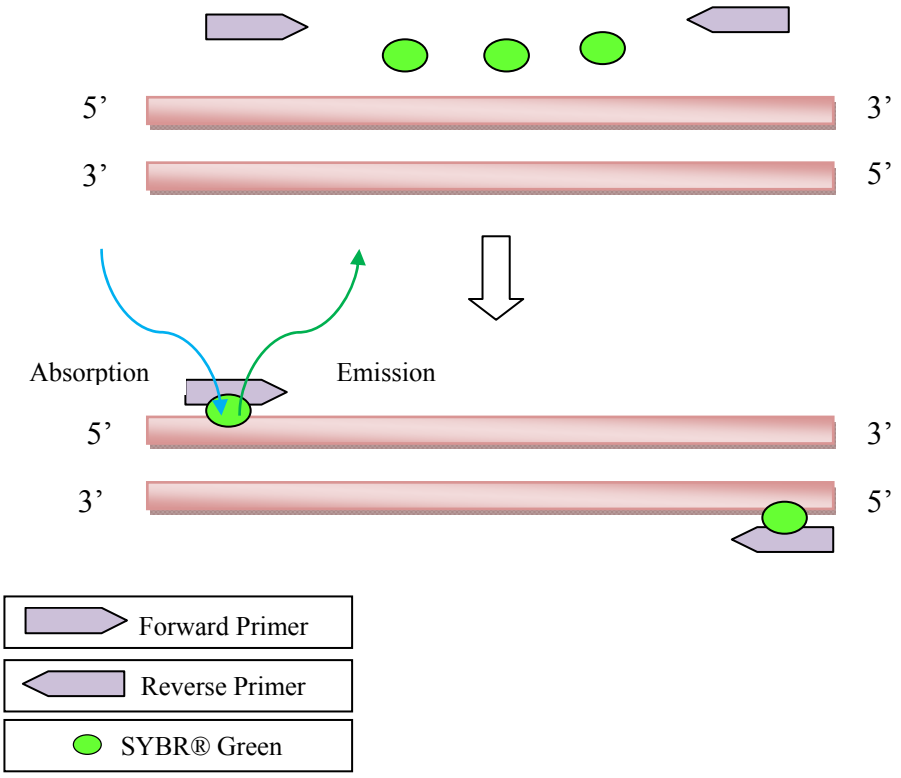

FIG. 1. SYBR® Green. The forward and reverse primers bind to the respective DNA strand. SYBR® Green binds which absorbs blue light at λ = 488nm and emits green light at λ = 522nm.

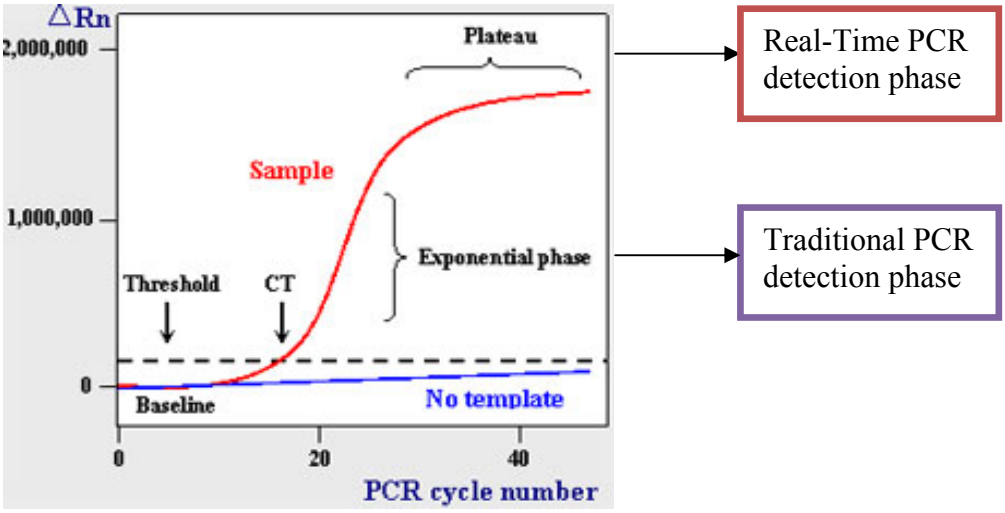

FIG. 2. Real-Time PCR Graph. The horizontal axis is the PCR cycle number while the vertical axis is the copy number (DNA product). The threshold line is set at the copy number at which fluorescence is observed to increase above the background level. The CT value is the copy number at which fluorescence crosses the threshold value. Source < http://www.rt-pcr.com/>

1.3 DNA extraction, purity and concentration determination

The presence of natural compounds, such as polysaccharides, fats, carbohydrates and proteins in samples may interfere with DNA extraction and affect down-stream processes including PCR amplification (15). As a result, these inhibitory substances must be removed from DNA-containing sample during extraction. Nature of the DNA extraction kits vary in their removal efficiencies and this often depends on the DNA-containing sample being tested. Traditionally, a spectrophotometer is used to determine DNA purity by measuring the intensity of absorbance at 260nm and 280nm (2). An A 260: A 280 ratio between 1.8-1.9 indicates highly purified DNA; whereas, a ratio less than 1.8 indicates protein contamination (2). Unfortunately, co-purification of acidified polysaccharides, such as DSS cannot be detected using a spectrophotometrically. In this study, DSS was observed to cause inhibitory effects on qPCR, and thru spiked DSS

experiments, concentration of co-purified DSS which causes inhibition in qPCR was determined.

1.4 Selecting target gene

In order to determine the abundance of specific members of the intestinal microbiota using qPCR, an appropriate target gene/sequence must be selected (9). The target gene should have variable and conserved regions discrimination of broad taxonomic levels, and provide annealing sites that enable the PCR primers, respectively (9). The 16S rRNA gene is most commonly used gene target for a number of reasons. It is universally conserved and exhibits low sequence degeneration.

CHAPTER II

METHODS

2.1 Collection and storage of fecal material

Our Animal Use Protocol (AUP) was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) prior to initiation of our study (Fig. 3). Ten Weanling Sprague-Dawley mice were used as subjects of these, six served as negative control and four were administered with 3% DSS through their drinking water for 48 hours. A 14-day period was present before the second course of DSS was administered (Fig. 3). The mice were kept in individual hanging cages and under a 12-hour light/dark cycle. A paper sheet was placed under each cage to collect feces; papers were replaced. There were six collection events and fecal samples were collected every 3 hours during the 24-hour collection period.

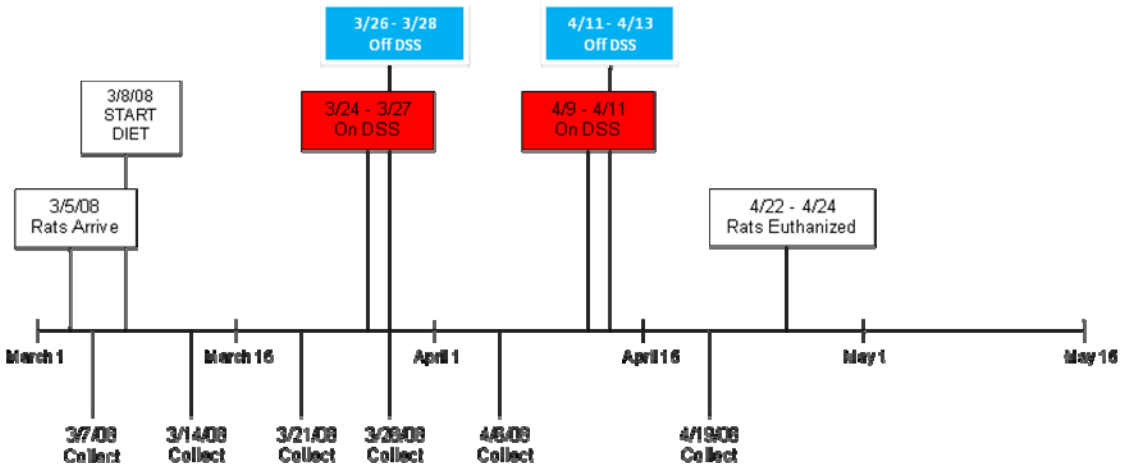

FIG. 3. Fecal Sample Collection Timeline.

Fecal samples were collected in pre-labeled 15ml tubes and stored at -80°C. Fecal samples were always kept on ice

2.2 DNA extractions

For each collection event, the weight of the fecal pellets was measured prior to extraction and recorded. 5ml of 10mM Tris (pH 8) was added to the fecal pellets which were homogenized using Fast Prep®-24 in 15 ml tubes. 0.5ml of fecal slurry was used for DNA extraction while the rest was frozen at -80°C freezer for future analysis. Bacterial DNA was extracted from homogenized fecal samples using the FastDNA®SPIN Kit for Soil according to manufacturer's protocol. In brief, samples were incubated for 5 minutes at 55°C to increase DNA yield. The NanoDrop™ 1000 spectrophotometer (TheromoScientific) was used to determine DNA concentration and purity at 260/280 nm. An aliquot of each sample was diluted to for 5ng/µl in DNase/Pyrogen-Free Water (DES).

2.3 Quantitative real-time polymerase chain reaction

SYBR® Green PCR reactions (25µl) were carried out in BIO-RAD iQ 96-well reaction plates in a BIO-RAD iCycler IQ5. PCR mixtures were composed of 12.5µl Quanta B-R SYBR® Green SuperMix for iQ*, 1µl of 5ng/µl DNA template, 9.5µl sterile Nanopure water, and 1µl of each 25µM Lac primer used for a final volume of 25µl (TABLE 1). *Lactobacillus*-group-specific primers Lac1 (**5'AGCAGTAGGGAATCTTCCA-3'**) and Lac2 (**5'ATTYCACCGCTACACATG-3'**) were used in the reaction. Each sample was

assayed in triplicates. Positive control reactions contained *L. acidophilus* (NCFM) as DNA template while the negative control reactions contained an equal volume of autoclaved Nanopure water. The cycling conditions used were 3min at 95°C, 45 cycles of 15 s at 95°C, and 45 s at 60°C. Amplification and data analysis were performed using the IQ™5 Optical System Software 2.0. Gene copies per PCR reaction were calculated using a standard curve generated for the assay.

TABLE 1. Components for qPCR

Substance	*Quantity*
1X Quanta B-R SYBR® Green SuperMIX	12.5µl
25µM Lac 1 Primer	1µl
25µM Lac 2 Primer	1µl
DNA Template (5ng/µl)	1µl
Autoclaved, Nanopure Water	9.5µl

2.4 Removal of inhibitory substances

When appropriate, residual DSS was removed using the Fast DNA® Spin Kit for Soil on spiked DSS fecal material and control fecal material lacking DSS. In order to determine the effects of DSS in relation to concentration, part of the extracted DNA was analyzed using qPCR, while the other part was further purified using Qiagen Dneasy Blood and

Tissue Kit and thereafter processed using qPCR (FIG. 4). The conditions used for qPCR were same as before. DSS concentration of 3mg, 0.3mg, 0.03mg, 0.003mg and 0mg were dissolved in 100 µl water. The water replaced 100µl of the 900µl (1/9) sodium phosphate buffer used in the extraction process. For each tested concentration, n=3. The assay was repeated with DSS concentration of 300mg, 30mg, 3mg, 0.3mg and 0mg. Thereafter, Qiagen Dneasy Blood &Tissue Kit was used to remove DSS from the samples in the study.

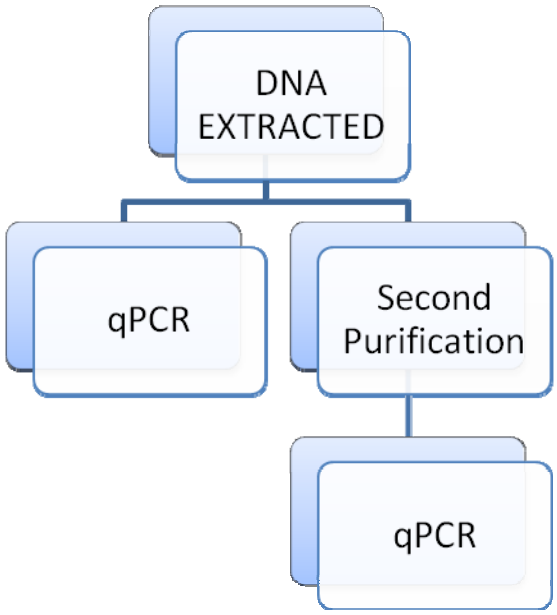

FIG. 4. Purification Process.

CHAPTER III

RESULTS AND DISCUSSION

The condition of human UC was mimicked in rodents using DSS in order to study its effects on the *Lactobacillus* population in the GI tract. *Lactobacillus* is part of the human intestinal microbiota; and a high count is considered to be beneficial to the host. Preliminary studies conducted on a rat's fecal samples showed no significant difference within one sitting of pellets but, a difference was observed in the pellets collected during different sittings within a collection event. In order to obtain a representative sample of all the organisms present within the 24-hour period collection, one pellet from each time point was combined into a 2ml tube to make a master mix. This was to eliminate intra-day variation and allow inter-day variation. qPCR was used to amplify 16S rRNA genes and detect bacterial counts of *Lactobacillus*. However, the two time points during which DSS was administered, day 21 and day 35, failed to amplify. C_t values > 30 indicated there was a significant inhibition and Ct value < 30 indicated successful amplification. We suspected that DSS had co-purified with DNA and was interfering in qPCR. Relative to the illustration (FIG.5), DSS time points show significantly lower copy numbers. Due to the observed qPCR inhibition from the DSS- treated time points, another purification step was performed in efforts of removing DSS.

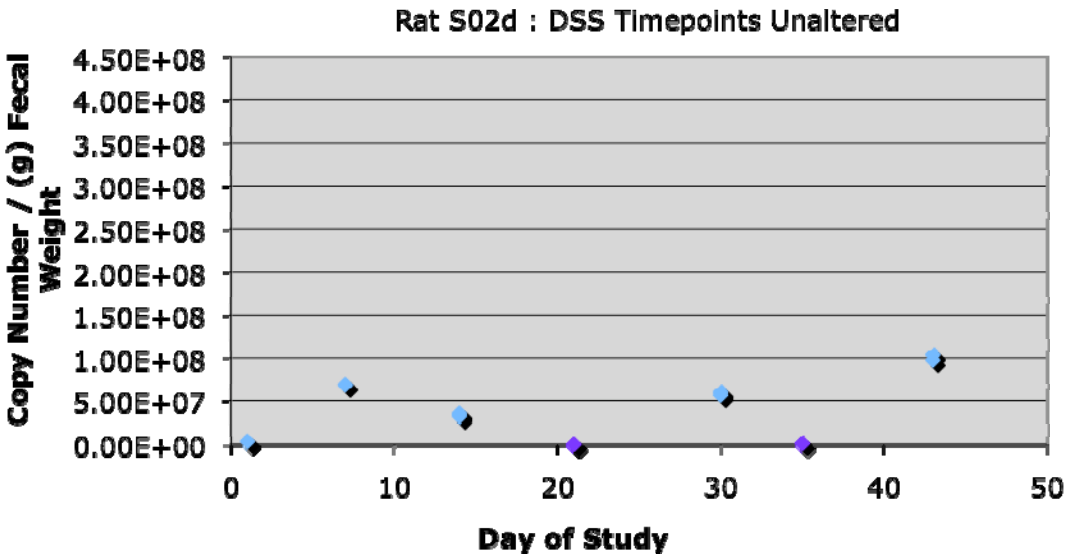

FIG. 5. Unaltered DSS Time-Points. During day 21 and day 35 when DSS was administered, a low copy number/g fecal weight is observed.

3.1 DSS spiking experiment

To determine if DSS was indeed able to inhibit qPCR, DSS was spiked into 0.5g fecal samples at concentrations of 3, 0.3, 0.03, 0.003 and 0mg. Plate 1 used the non-Qiagen treated DNA template while plate 2 used the DNA obtained following the Qiagen purification. Amplification was observed in Plate 1 (FIG A.1) for concentrations of 0.03, 0.003, and 0mg whereas; concentrations of 3 and 0.03mg failed to amplify or amplified extremely late (TABLE A.1). The high C_t values observed display DSS is inhibitory at concentrations of 3 and 0.3 mg and could be co-eluted into the DNA samples during the first purification step, which was part of the extraction step. The samples were cleaned using the Qiagen Kit to determine if a difference in amplification patterns would result. The amplification chart (FIG A.2) shows amplification of all

samples including concentration of 3 and 0.3mg, which were previously observed to be inhibited. TABLE A.2 shows the C_t values for each sample. The C_t values of the purified samples improved therefore it can be determined that initially co-purified DSS can be removed by processing samples through another purification cycle.

The second assay designed with higher concentrations of DSS was used to determine if a certain concentration of DSS would remain inhibitory irrespective of purification. Concentration of 300, 30, 3, 0.3 and 0mg were added to fecal samples. Amplification for all samples containing DSS was either completely inhibited or was observed extremely late which is not considered significant amplification. Samples without DSS or 0mg of DSS are observed to amplify (FIG A.3). TABLE A.3 presents high C_t values obtained from the different concentrations of DSS. The samples were purified and concentrations of 30, 3, 0.3 and 0mg of DSS amplified successfully (FIG A.4) with low C_t values (TABLE A.4). Samples containing 300mg showed better C_t values compared to TABLE A.3, which contained C_t values of extracted samples, which had not undergone another purification cycle. However, the C_t values do not indicate significant amplification. Samples from the original study were then purified using the Qiagen Kit and an increase in copy numbers was observed for day 21 and day 35 when DSS was administered, which indicates successful amplification (FIG 6). Therefore, it can be stated that our samples contained less than 300mg but more than 0.3mg of DSS since they were able to amplify after the second round of amplification but not after the first.

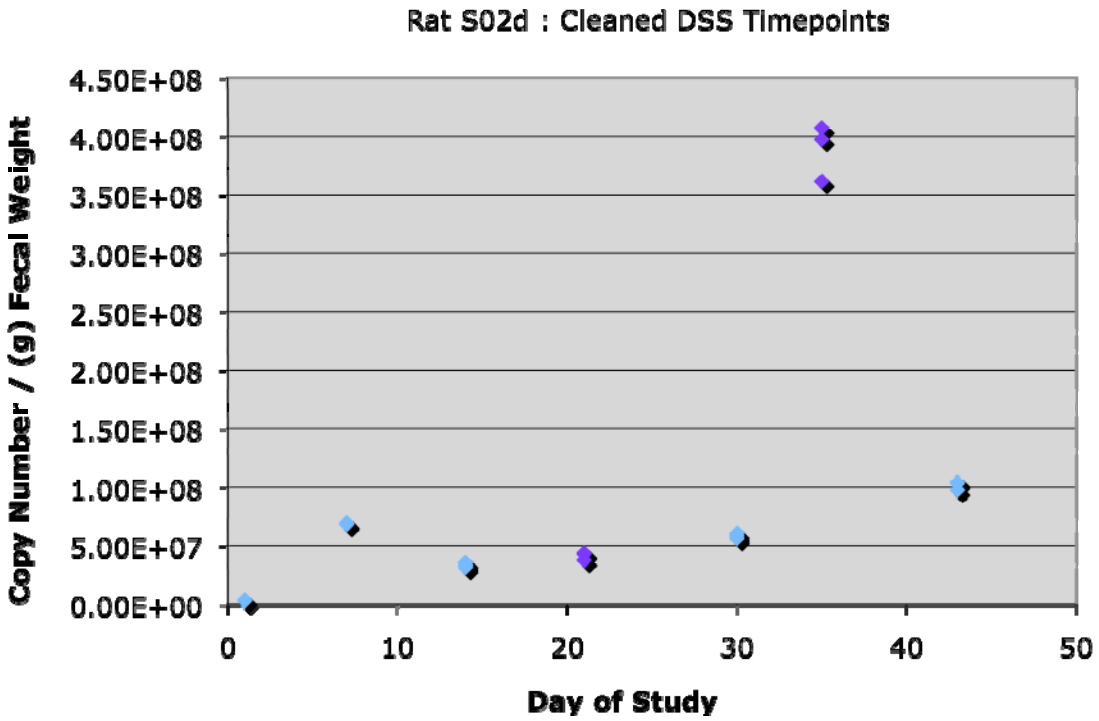

FIG. 6. Altered DSS Time-Points. The co-purified DSS in the DNA samples from the fecal samples was processed using another purification cycle and the copy number/ g fecal weight significantly increased on day 21 and day 35 when DSS was administered.

3.2 Heparin and DSS comparison

DSS was related to one of the known PCR inhibitors called heparin. Heparin, widely used as an anticoagulant, has been demonstrated to competitively inhibit DNA polymerase including Taq polymerase (5, 6, 13, 16). The chemical structures of both molecules were compared for similarities and it was found that both molecules are acidic polysaccharides, anionic polysaccharides, contain sulfate and alcohol groups, have an ether linkage connecting the disaccharides, and both are similar in structure compared to DNA. The observed differences include the presence of carboxylic acid and amide in heparin but, not in DSS. Also, there are three sulfate groups present on the heparin

disaccharide compared to two sulfate groups on DSS. It has previously been stated that presence of natural compounds such as polysaccharides, fats, carbohydrates and proteins in samples may impede DNA extractions and affect PCR amplification (7). Due to heparin being a strong anionic polysaccharide, it has been suggested that heparin interferes with PCR possibly from a resulting conjugation between heparin and Taq DNA polymerase (16). The importance of discussing sulfate groups is due to their reported inhibitory role in heparin (1, 13). Studies have showed that the amount of sulfate concentration present in heparin is responsible for causing inhibition (1, 13). Heparinase I can reverse the inhibitory characteristics of Heparin by cleaving the polysaccharide into disaccharide units therefore decreasing the amount of sulfur (1, 13). Heparin to DNA ratios have been tested and reported that the degree of inhibition is directly correlated with the sulfate content (1). In comparison, DSS was observed to be inhibitory at certain concentrations in our study. Also, in heparin inhibition can be overcome by using heparinase and similarly the Qiagen purification kit used in our study showed amplification on previously inhibited samples. Therefore it can be hypothesized that DSS inhibits Taq polymerase in qPCR via a competitive mechanism for the DNA template strand in similarity to Heparin. However, further studies will be required to verify this hypothesis.

CHAPTER IV

SUMMARY AND CONCLUSION

DSS is a well established model used to reproduce human UC in rodents in order to study the effects of inflammation on the GI microbiota. DNA extracted from fecal samples using the FastDNA®SPIN Kit for Soil showed reduced amplification, but only at DSS-treated time points in the study. We show that DSS acts as a PCR inhibitor and developed methods to optimize its renewal from fecal samples. Therefore, it was hypothesized that DSS could be a potential PCR inhibitor. This hypothesis was tested using DSS-spiked fecal material. DNA was again extracted using FastDNA®SPIN Kit for Soil. A part of the DNA was analyzed using qPCR, while the other part underwent another purification step using Qiagen Dneasy Blood and Tissue Kit. DNA samples containing 0.03mg or less co-purified DSS do not inhibit amplification. Concentrations of 30mg and 3mg DSS show successful amplification only post qiagen-purification. 300mg DSS does not significantly amplify even after qiagen-purification. It is believed that DSS inhibits qPCR by competing with DNA for the Taq polymerase binding/active sites, as seen with another acidic polysaccharide Heparin.

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APPENDIX

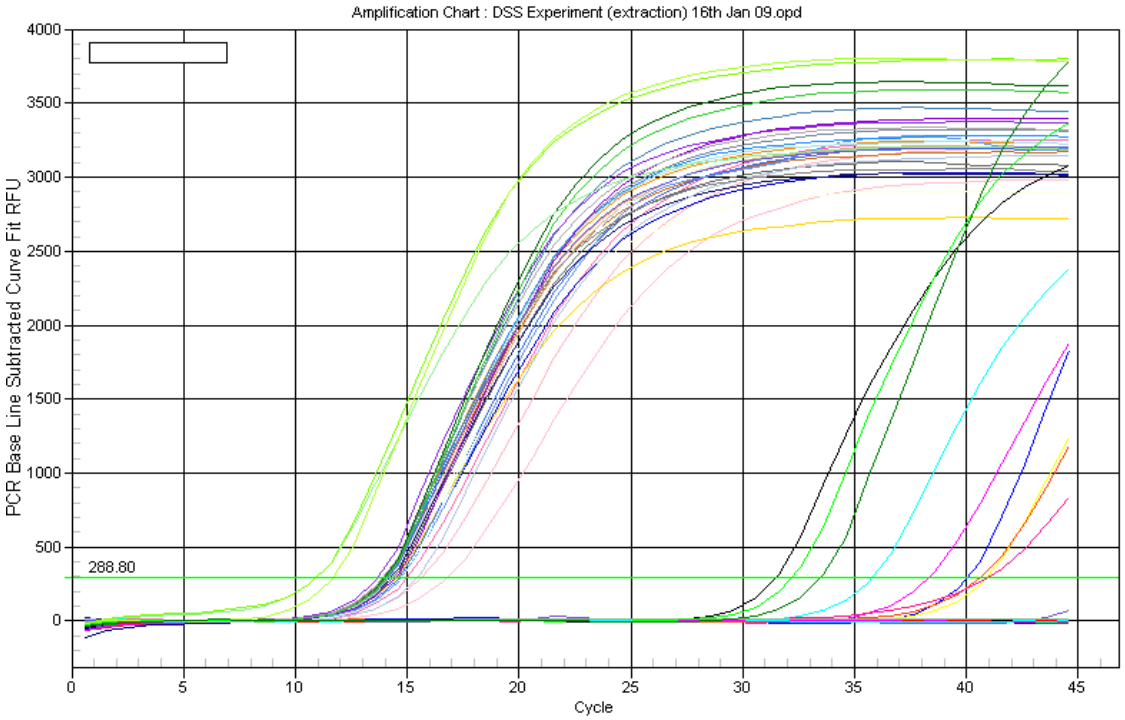

FIG A.1. Amplification Chart of Extracted Samples in First Assay. Samples with concentration of 3mg and 0.3 mg either failed to amplify or amplified really late which is not considered significant amplification. Negative control amplified really late and positive control amplified early as expected. Concentrations of 0.03 and 0.003 mg showed successful amplification.

TABLE A.1 C_t Values of Extracted Samples in First Assay

The sample type in the table refers to sample identification in which the number represents concentration of DSS and the letter represents n which equals 3 therefore, A, B, and C are used. Each sample was run in triplicates. $C_t > 30$ indicate significant inhibition whereas, $C_t < 30$ indicates successful amplification. N /A indicates not amplified

	1	2	3	4	5	6	7	8	9	10	11	12
SampleType			3-A	3-A	3-A	3-B	3-B	3-B	3-C	3-C		
ThresholdCycle			N/A	N/A	N/A	39.97	38.26	N/A	N/A	N/A		
SampleType			3-C	0.3-A	0.3-A	0.3-A	0.3-B	0.3-B	0.3-B	0.3-C		
ThresholdCycle			40.84	N/A	35.78	N/A	40.59	40.91	N/A	14.99		
SampleType			0.3-C	0.03-A	0.03-A	0.03-A	0.03-B	0.03-B	0.03-B	0.03-C		
ThresholdCycle			16.77	15.65	14.01	13.92	14.07	14.20	14.41	14.49		
SampleType			0.03-C	0.03-C	.003-A	.003-A	.003-A	.003-B	.003-B	.003-B		
ThresholdCycle			14.71	14.61	14.72	14.05	N/A	14.75	14.32	14.20		
SampleType			.003-C	.003-C	.003-C	0-A	0-A	0-A	0-B	0-B		
ThresholdCycle			13.99	14.13	N/A	14.14	13.66	14.43	14.08	15.41		
SampleType		0-B	0-C	0-C	0-C							
ThresholdCycle		13.90	13.88	13.95	14.01							
SampleType			POS	POS	POS	NTC	NTC	NTC				
ThresholdCycle			10.90	10.92	11.65	31.42	32.18	33.46				
SampleType												
ThresholdCycle												

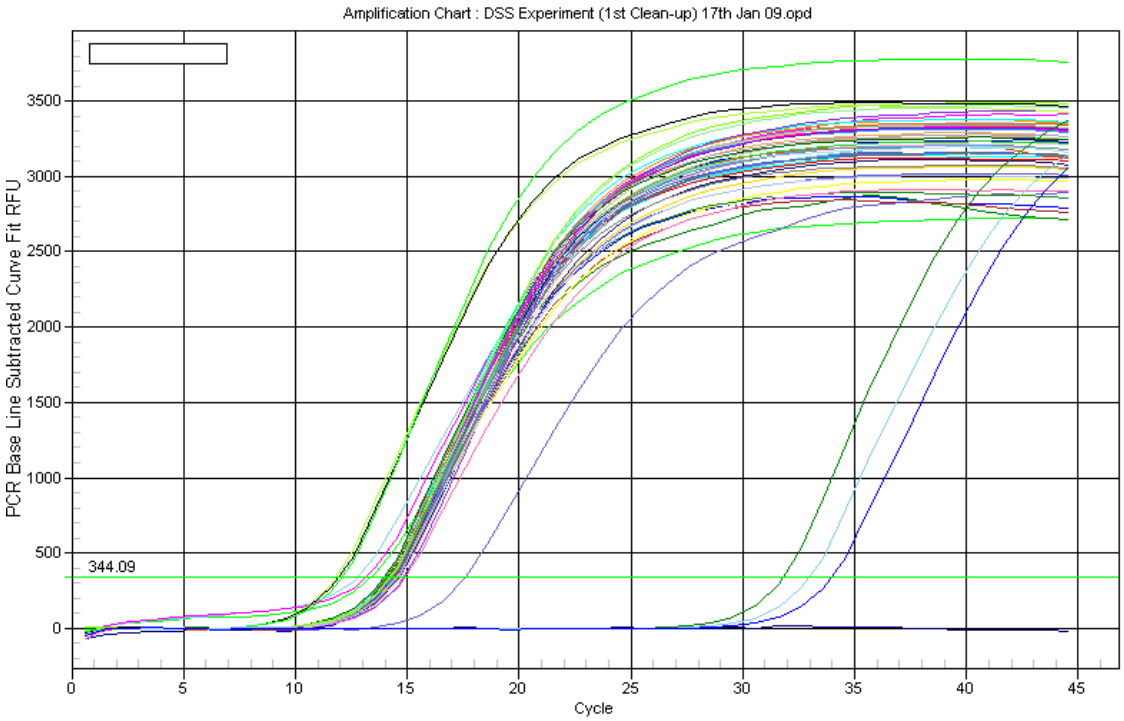

FIG A.2. Amplification Chart of Qiagen-Purified Samples in First Assay. Samples from all concentrations showed successful amplification. Samples that were previously inhibited showed amplification at significant C_t values. Negative control amplified really late and positive control amplified early as expected.

TABLE A.2 C_t Values of Qiagen-Purified Samples in First Assay

The sample type in the table refers to sample identification in which the number represents concentration of DSS, (*) represents Qiagen-purified samples and the letter represents n which equals 3 therefore, A, B, and C are used. Each sample was run in triplicates. $C_t > 30$ indicate significant inhibition whereas, C_t

	1	2	3	4	5	6	7	8	9	10	11	12
SampleType			3*-A	3*-A	3*-A	3*-B	3*-B	3*-B	3*-C	3*-C		
ThresholdCycle			13.39	14.13	12.77	14.37	13.14	14.30	N/A	17.60		
SampleType			3*-C	0.3*-A	0.3*-A	0.3*-A	0.3*-B	0.3*-B	0.3*-B	0.3*-C		
ThresholdCycle			14.24	14.05	14.03	13.98	14.16	14.30	14.31	14.93		
SampleType			0.3*-C	0.3*-C	0.03*-A	0.03*-A	0.03*-A	0.03*-B	0.03*-B	0.03*-B		
ThresholdCycle			14.71	14.49	14.40	14.21	14.38	14.39	14.49	14.48		
SampleType			0.03*-C	0.03*-C	0.03*-C	0.003*-A	0.003*-A	0.003*-A	0.003*-B	0.003*-B		
ThresholdCycle			14.30	14.31	14.44	14.21	13.99	13.93	14.22	14.65		
SampleType			0.003*-B	0.003*-C	0.003*-C	0*-A	0*-A	0*-A	0*-B	0*-B		
ThresholdCycle			14.58	14.60	14.59	14.20	14.86	14.39	14.42	14.56		
SampleType			0*-B	0*-B	0*-B	0*-C	0*-C	0*-C				
ThresholdCycle			14.32	14.01	13.97	14.29	14.24	14.21				
SampleType			POS	POS	POS			NTC	NTC	NTC		
ThresholdCycle			11.80	11.93	12.07			31.82	32.98	33.98		
SampleType												
ThresholdCycle												

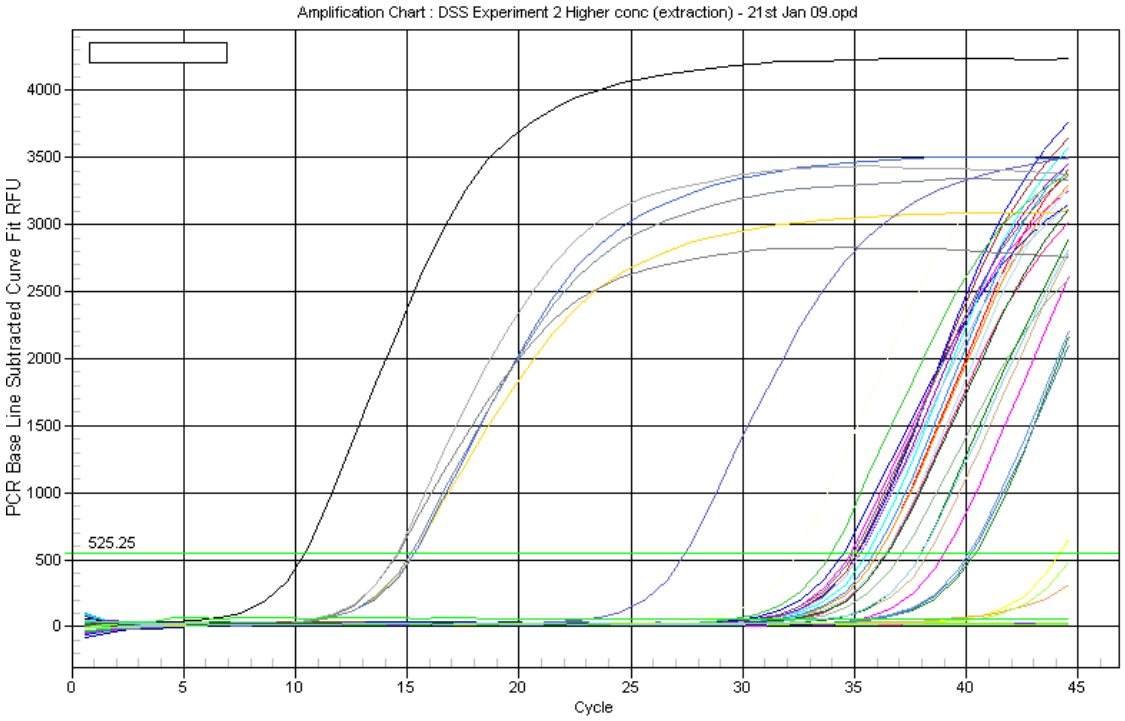

FIG A.3. Amplification Chart of Extracted Samples in Second Assay. Samples lacking DSS or 0mg were the only observed amplified samples. Concentrations of 300, 30, 0.3 and 0.03 were found to be inhibitory. They either lacked amplification or amplified really late

TABLE A.3 C_t Values of Extracted Samples in Second Assay

The sample type in the table refers to sample identification in which the number represents concentration of DSS and the letter represents n which equals 3 therefore, A, B, and C are used. Each sample was run in triplicates. $C_t > 30$ indicate significant inhibition whereas, $C_t < 30$ indicates successful amplification. N/A indicates not amplified.

	1	2	3	4	5	6	7	8	9	10	11	12
SampleType			300-A	300-A	300-A	300-B	300-B	300-B	300-C	300-C		
ThresholdCycle			N/A	N/A	N/A	35.11	39.01	35.06	N/A	N/A		
SampleType			300-C	30-A	30-A	30-A	30-B	30-B	30-B	30-C		
ThresholdCycle			N/A	40.36	N/A	36.05	N/A	36.40	34.80	N/A		
SampleType			30-C	30-C	3-A	3-A	3-A	3-B	3-B	3-B		
ThresholdCycle			N/A	N/A	N/A	36.06	N/A	38.29	32.47	15.33		
SampleType			3-C	3-C	3-C	0.3-A	0.3-A	0.3-A	0.3-B	0.3-B		
ThresholdCycle			34.49	15.33	40.07	35.87	35.60	N/A	34.93	N/A		
SampleType			0.3-B	0.3-C	0.3-C	0.3-C	0-A	0-A	0-A	0-B		
ThresholdCycle			40.19	15.16	37.09	36.34	N/A	35.24	27.41	35.22		
SampleType			0-B	0-B	0-C	0-C	0-C					
ThresholdCycle			14.58	14.54	36.44	33.91	N/A					
SampleType			POS	POS	POS			NTC	NTC	NTC		
ThresholdCycle			N/A	N/A	10.44			N/A	37.96	37.96		
SampleType												
ThresholdCycle												

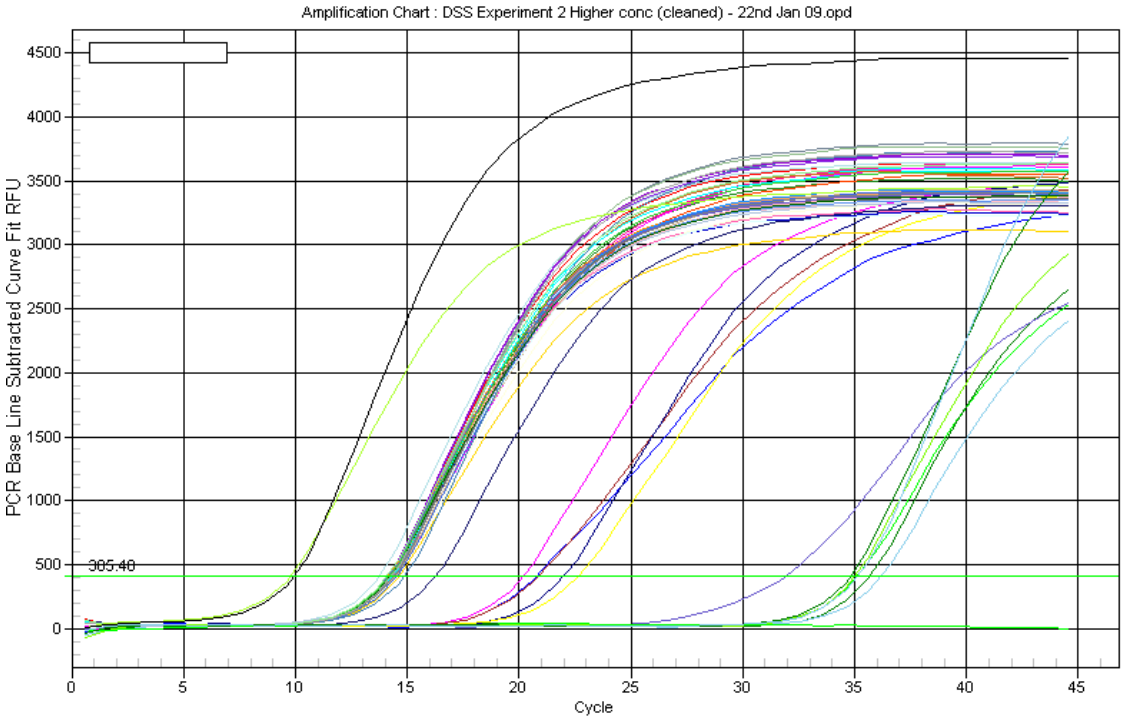

FIG A.4 Amplification Chart of Qiagen-Purified Samples in Second Assay. Samples containing DSS at concentrations, 30, 3,and 0.3mg showed successful amplification. Sample containing 300mg DSS showed better Ct values after Qiagen-purification however, they do not indicate significant amplification. Negative control amplified really late and positive control amplified early as expected.

TABLE A.4 C_t Values of Qiagen-Purified Samples in Second Assay

The sample type in the table refers to sample identification in which the number represents concentration of DSS, (*) represent Qiagen-purified, and the letter represents n which equals 3 therefore, A, B, and C are used. Each sample was run in triplicates. $C_t > 30$ indicate significant inhibition whereas, $C_t < 30$ indicates successful amplification.

	1	2	3	4	5	6	7	8	9	10	11	12
SampleType			300*-A	300*-A	300*-A	300*-B	300*-B	300*-B	300*-C	300*-C		
ThresholdCycle			35.10	35.66	36.16	20.75	20.17	20.85	21.98	31.90		
SampleType			300*-C	30*-A	30*-A	30*-A	30*-B	30*-B	30*-B	30*-C		
ThresholdCycle			22.67	14.29	14.17	14.10	14.13	14.15	14.52	14.08		
SampleType			30*-C	30*-C	3*-A	3*-A	3*-A	3*-B	3*-B	3*-B		
ThresholdCycle			14.20	14.23	14.28	14.10	14.26	14.44	14.52	14.63		
SampleType			3*-C	3*-C	3*-C	0.3*-A	0.3*-A	0.3*-A	0.3*-B	0.3*-B		
ThresholdCycle			14.26	14.56	14.38	14.26	14.30	14.37	14.34	16.27		
SampleType			0.3*-B	0.3*-C	0.3*-C	0.3*-C	0*-A	0*-A	0*-A	0*-B		
ThresholdCycle			14.86	14.35	14.25	13.73	14.07	14.08	14.12	14.20		
SampleType			0*-B	0*-B	0*-C	0*-C	0*-C					
ThresholdCycle			14.40	14.26	14.20	14.16	14.05					
SampleType												
ThresholdCycle												
SampleType			POS	POS	POS		NTC	NTC	NTC			
ThresholdCycle			34.99	9.77	9.93		N/A	34.80	35.13			

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